SOME INTROSPECTIONS ON MOLD METABOLISM[1]

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Some two hundred years ago, it is said (7), Albrecht von Haller, famous Swiss scientist, described fungi as "a mutable and treacherous tribe." However limited the stimuli which induced von Haller to make this observation in those beginning days of the study of these organisms, modern students of the subject, with a vast background at their disposal, can only marvel at and attest to his astuteness. Since von Haller was mainly a plant taxonomist and animal physiologist, and since his time was well before the inception of biochemical studies on molds (indeed, biochemistry in general), first firmly initiated by Raulin and Pasteur more than a century later, there is little doubt that it was the gross cultural aspects of fungi he was concerned with when he made his grim conclusion. He could have had no idea of the tremendously wide application his verdict was to have on the then as yet unopened fields of physiological and biochemical and, in general, metabolic aspects of fungi. And yet the same kind of frustration implicit in von Haller's succinct verdict is only too well known to the investigator of metabolism,—and with variations far beyond the scope of that versatile scientist's penetrating imagination.

Time and mycologists have amply confirmed the incessant tendency of filamentous fungi toward spontaneous change, and this mutability has become recognized as one of the outstanding traits of this group of organisms. Doubtless such mutability holds also for all microörganisms. Its recognition first in the fungi probably has two explanations: the gross size of these organisms in culture makes changes easily discernible, and structural differentiation of the organism into diverse morphological parts increases greatly the chances for visible deviation from parent cultures or from the norm.

It was probably the industrial or applied microbiologist who was largely responsible for the emergence of the idea that in fungi there exists a mutability of even greater range and diversification than that observable by superficial inspection. Detailed studies through the years on organisms of potential practical interest from the standpoint of their metabolic ("fermentation") activities have led to the following what may be considered as axioms in microbial metabolism, and especially in relation to molds:

1. The individual progeny from any culture of single spore origin may vary within wide limits in regard to the performance of any given biochemical activity, despite the fact that all may be morphologically indistinguishable. This phenomenon, known as strain specificity,[2] is not, with relatively few exceptions,

[1] The material in this article represents substantially the contents of a chapter from a book on mold metabolism under preparation by the author.

[2] Incidentally, it may be pointed out that this conclusion, like so many "new" concepts and ideas in microbiology, and other sciences as well, turns out to be merely a rediscovery of a feature first recognized by a past master. In this case it was the genius of Pasteur who

evident from simple inspection, but is revealed only by chemical analysis. The degree to which this kind of mutability is so fundamental in theoretical and practical microbial metabolism is emphasized by the consideration that in addition to the range occurring with any one function, it must also occur individually with respect to every one of the host of biochemical reactions the organism is capable of effecting. Thus the cells in any one culture are far from being homogeneous physiologically.

When progeny with weaker power to effect a given biochemical function, get the upper hand in an initially potent culture, as they may in the course of continued successive transfer, the ultimately weakened culture is said to have undergone "physiological degeneration". Viewed in this way, the physiological potentialities of any culture are always changing during periods of active growth.

With such differences possible within the progeny of any one culture, it scarcely needs to be emphasized how great differences may be expected between morphologically indistinguishable strains of diverse origins, such as isolates from natural sources, or from different stock culture collections. Apart from quantitative differences, these often are even qualitatively different.

2. In addition to the above, there is another kind of variation in progeny, and this is latent. Two morphologically indistinguishable strains compared metabolically under any one set of conditions may, within experimental limits, respond so nearly alike that they might be considered physiologically indistinguishable. Yet, tested together under another set of conditions, gross differences in metabolic behavior may become evident. There are at this time few examples of this type to call upon among the fungi, but the data of Schulz (13) make a neat instance. This author studied extensively the proximate chemical composition of the cell material of *Aspergillus niger* and found that the compositions of two different strains were virtually indistinguishable when cultivated on a certain basal medium. The addition of a few ppm of zinc ion to this medium caused marked changes in composition of the mycelium, but the changes were strikingly different in the two organisms, so that there was no question that two different individuals were involved.

It is interesting to note that this procedure of discerning metabolic differences between morphologically indistinguishable organisms, actually forms, to a great extent, the basis of diagnostic bacteriology. The principle is the same,

in 1876 fully perceived, appreciated and even defined strain specificity, only he called it "le polymorphisme physiologique" (12a). In previous works the present writer has, unknowingly, been equally guilty with many others of this particular "rediscovery", full credit for which, we hope, will henceforth be ascribed exclusively to whom it belongs—that French savant.

"On pourrait croire que toutes les variétés de *mucor* sont propres à donner le genre de levûre dont nous venons de parler. Il n'en est riên. C'est encore une preuve frappante des différences physiologiques profondes que peuvent offrir des formes de végétation pourtant si voisines que les classifications botaniques sont contraintes de les rapprocher autant qu'il est possible. Déjà les *mycoderma vini* et les levûres alcooliques proprement dites, si semblables de formes et de développements qu'on les jugerait identiques, au moins dans l'état de nos connaissances, et si différents physiologiquement, donnent de ce fait un exemple extraordinaire" (12b).

—two organisms behave alike in their response to different physiological tests, and differentiation becomes possible only after a sufficient number of tests are applied so that eventually the sought-for difference in response materializes. In bacteriology, such latent properties, ultimately revealed, provide the basis of species differentiation, and sometimes even genus separation. Fortunately, the systematics of fungi is firmly based on morphology, for if latent physiological characters were employed as a means of species differentiation, the numbers of species would truly be infinite.

The reasons why such diagnostic techniques are workable with the bacteria and not with fungi, are manifest to the microbiologist. Diagnosis or differentiation is based largely upon the plus or minus ability of bacteria to attack certain individual (saccharidic) compounds as energy sources. Similar exhaustive differential testing has not been done with many strains of any given species of fungus because such a feature has little diagnostic value in a system based exclusively on morphological criteria for identification. The important feature of mold strains is the intensity with which individual strains carry out particular biochemical transformations. As brought out in the next paragraph, fungi are extremely sensitive and unstable in this respect, whereas the bacteria as a class are much more immune to environmental circumstances and can generally be counted on to effect a stable and reproducible dissimilatory metabolism.

3. The first two axioms of variation are based on differences inherent between different fungus individuals, and there is good reason for believing that they are of genetic or nuclear origin. This is, then, genetic variation. The third axiom is predicated on a different kind of variation, one quite apart from genetic differences between individuals. This relates to the extreme susceptibility of the physiological potentialities of any given fungus culture—which, because of the enormous number of cells, may be considered collectively to function as an individual—to relatively slight alterations in environmental factors. We may designate this as "response variation", and the discussion throughout this article deals with responses in a metabolic sense. The term "physiological variation" is often employed, but it does not have the connotation that the former term does in excluding genetic phenomena and in limiting the meaning to environmental sensitivity.

The unique and tremendous response differences, both quantitative and qualitative, obtainable with any single fungus culture as a result of imposed cultural conditions are so common an experience and so well known to the microbiologist that it is not worthwhile going into specific detail here, as it is more the fundamental cause we are concerned with than the effects. However, for an orientation of the reader there may be cited biochemical characters such as amount and composition of cell matter synthesized, formation of extracellular enzymes, production of low molecular weight synthetic compounds (e.g., antibiotics, vitamins, pigments, etc.), accumulation of sugar split products (carboxylic acids), carbon dioxide evolution, and so on. Generally all of these are in balanced interrelation, a change in one usually being reflected by changes in others.

In passing, it may be mentioned that the alterations in cultural conditions which can induce most significant metabolic changes are often so slight that they are exceedingly difficult if not impossible to control fully, despite rigorous precautions. This accounts for the difficulties in reproducibility of results between different laboratories employing identical cultures, and even between different trials in the same laboratory (penicillin yields still fluctuate within disturbingly wide extremes).

The remainder of this discussion, and the next three sections especially, is an attempt to analyze some of the intimate aspects of mold carbohydrate metabolism with the object of synthesizing a coördinating and interpretative scene from episodes which, it seems to the writer, have a bearing on Axiom *3* above. If they are critically evaluated in this manner, the author will have achieved his objective, namely, the stimulation of thinking towards generalized concepts in the field of mold metabolism which hitherto has been deplorably neglected in this respect. Admittedly, certain of these ideas may be considered by some to be premature, distorted, and incomplete. If my colleagues and others consider this essay too speculative for their scientific natures, my defense rests on two licenses: namely, the title of this essay, and the quotation of A. V. Hill which Kluyver used in defense of his (Kluyver's) speculations and attempt at synthesis of the field of bacterial metabolism sixteen years ago: "It is dangerous to speculate too far, but it is foolish not to speculate at all."

EFFICIENCY OF CELL SYNTHESIS OF MOLDS IN RELATION TO NATURAL ENVIRONMENT

It has already been stated that the metabolism of any given mold culture is dependent upon environmental conditions, and that it can be made to fluctuate between extremely wide limits so that the change may actually take on the appearance of a qualitative difference. Indeed, it is a common event to have an organism produce no detectable amount of a particular metabolic product, and yet under different cultural conditions, produce that very substance abundantly. Finally, there is the situation in which, on the one hand, one kind of product is produced, and, on the other, a totally different product. What is the explanation of such behavior? While no single interpretation may provide the full story, it seems as though sufficient experimental and circumstantial evidence is available to provide a start on this intriguing question.

In addition to the above mentioned major metabolic differences between filamentous fungi (molds) and bacteria, it may be pointed out that with possibly occasional isolated exceptions there are no anaerobic molds. It is doubtful if obligately anaerobic molds exist. Indeed, there is general concurrence with the idea that molds are highly oxidative organisms. This is not say that molds with not metabolize carbohydrates anaerobically (fermentation), but this activity is accomplished by preformed cell material.

Even though reduced products are formed in aerobic cultures, e.g., ethanol, they are without a doubt formed by cells deficient in oxygen. Under the usual conditions of cultivation, some always are. It is questionable if molds can de-

velop, that is, *grow* at a significant rate in the complete absence of molecular oxygen. Certainly such exceptions are exceedingly rare.

The possession by many molds of strong glycolytic mechanisms is, as seen below, the outstanding metabolic characterization of many of these organisms. On the other hand, possession of strongly aerobic metabolism has a profound implication for the economy of the mold organism insofar as utilization of its available energy source goes. It means that the organism has innately the ability to utilize most efficiently its substrate for growth and cell synthesis due to the fact that aerobic respiratory processes are the most efficient for liberating and utilizing, for multifarious cellular activities, the energy of the substrate which we shall assume is carbohydrate. Considering the conditions prevailing in nature, such as in the soil, the natural habital for molds, the idea of organisms possessing a type of metabolism which enables them to utilize substrate efficiently and to build up as much cell material as possible seems not unlikely.

Due to the rather frugal and precarious nutritional environment prevailing in the natural habitat of molds, it appears not unreasonable that they have become adapted to survival and existence under threshold nutritional conditions by their high efficiency of utilization of the limited energy source available.

The nutritional level of the soil must at most times be very low, except during those relatively isolated periods when fresh plant or animal residues are available. Even then in neutral soils the fast-growing bacterial, and to some extent actinomycetal, population accounts for the destruction of the bulk of the readily utilizable material. The fungi come in, then, as secondary invaders, utilizing certain of the more resistant components of the organic matter and the remains of other microbial cells. Thus, through the competition with the rest of the soil population for available foodstuffs and the slow utilization of resistant organic fractions in the soil, the molds may be thought of as having become adapted through natural selection (possibly preceded by mutation) to a highly effective economy in their prevailing environment, e.g., a low level or marginal nutritional state with respect to available energy and carbon source. Such an effectively economic metabolism might account for the survival of and the high number of fungi occurring in normal soils. This superior character could, then, be interpretable as a consequence of the failure of the organisms to waste energy in the form of products of anaerobic or aerobic metabolism, giving the efficient molds survival advantage over inefficient ones, which eventually would become extinct.

The important consequence of the efficiency concept is that maximum efficiency of energy utilization by the mold is attained only when the substrate-carbon is converted entirely into only two products, namely, the components of protoplasm and the inevitable CO_2. There is no evidence that molds form any dissimilation products under normal soil conditions, and in line with the foregoing, the only reasonable conclusion is that molds in soil oxidize these substrates entirely to CO_2 and H_2O, aside from that relatively high fraction converted into cell material. This concept probably has general application to the majority of aerobic microörganisms under the nutritional conditions

prevailing in soils most of the time,—complete conversion of substrate to CO_2, H_2O and cell material. Exceptions would be those relatively few cases where split products of certain complex naturally occurring compounds would accumulate. Such substances could not be attacked by the organism under any circumstances, and are not to be considered as dissimilation products, inasmuch as usually they consist of unchanged portions of the substrate molecules, rather than products arising from the substrate through transformations brought about by intermediary metabolism; and, in most cases, they resemble structurally the original substrate molecule. As an example, one might cite the oxidation of the side chain of an aromatic compound, leaving the ring structure intact. In any case some organism could be found to decompose the compound completely.

The inefficiency of anaerobic organisms in the utilization of substrate is a consequence of their leaving always a portion of the substrate in the form of metabolic reduction products, or expressed differently, they leave the major portion of the energy of the substrate in the form of organic metabolism products. Obviously, with less energy obtained, the growth efficiency is reduced. The same line of reasoning holds true in the case of metabolic products produced by molds as a result of aerobic consumption of carbohydrate, namely, organic acids and other excretion products. Energy left in the form of accumulated products of any kind, actually means reduced efficiency of energy utilization up to that state. In most cases the products may be further attacked after the original substrate is depleted, and their energy utilized (see later).

Hypothetical anaerobic (true) fungi probably could never survive competitively with the anaerobic bacterial population of the soil in the utilization of fermentable carbohydrates.

OVERFLOW AND SHUNT METABOLISM

Why then do molds produce from sugar large amounts of metabolic products other than cell material and CO_2, namely organic acids, carbohydrates, polyhydric compounds, etc.? The best explanation seems to be that the metabolism of the organism becomes deranged. It becomes so to speak, pathological. This pathological behavior is a direct result of the influence of abnormal environmental conditions.[3]

Of greatest importance is the carbohydrate concentration. Invariably laboratory media for the cultivation of fungi contain carbohydrates in concentrations far exceeding those that the organism ever would encounter in nature and to which the mold is adapted for maximum efficiency of utilization. This luxury of excess sugar sets off a chain of events culminating in faulty metabolism of the sugar as indicated by only partial utilization of the sugar molecule, leaving

[3] This general idea had already been cogently expressed for activities of soil bacteria by the venerable Sergei Winogradsky in 1928. He emphasized that the activities of soil bacteria in the laboratory are no criterion of their behavior in the soil complex. He called such laboratory cultures "domesticated hothouse organisms". Soil Sci. **25**, 37–43; **43**, 327–40 (1937).

incompletely oxidized products accumulating in the medium, usually indicated as organic acids, although other products may also accumulate outside and inside the cells. It would appear that the enzyme mechanisms normally involved in complete oxidation of the substrate become saturated, and the substrate molecules then are excreted and accumulate as such; or they are shunted to secondary or subsidiary enzyme systems which are able to effect only relatively minor changes in the substance, which then accumulates in its transformed state. The latter mechanism is by far the most common. The limiting or bottleneck enzyme systems are never those concerned with the initial stages of carbohydrate dissimilation, but are those which act on the substrate only after it has been brought through the stage of split products. When the rate of attack on the original sugar molecule is limiting, obviously subsequent enzymes in the chain can accommodate in the normal way all the raw split product available to them, no diversion results, and no waste dissimilation products ensue. However, when the rate of splitting the carbohydrate chain into smaller products proceeds faster than the subsequent enzymes can handle them, a metabolic shunt occurs, resulting in accumulation of waste products, or increase in other products produced only in small amounts normally. The inability to metabolize rapidly intermediates, which then are diverted through abnormal channels, has many analogies in general biochemistry. For example, in yeast fermentation, the enzymes normally giving rise to ethanol can by chemical treatment be made limiting or inoperative so that triose from sugar is diverted to glycerol instead of ethanol. In animal as well as microbial metabolism, carbohydrate nutrition above that required for basal metabolism is diverted to fat, which represents deposits of condensed sugar split products which accumulate as fat when the normal oxidation enzymes are surfeited.

In fact, the probability is good that metabolic shunts actually are the basis of the widespread practice of securing increased intensity of certain biochemical properties on the part of various organisms through mutation techniques, irradiation, etc. Especially has this objective been sought in connection with the biosynthesis of industrially important compounds,—penicillin, streptomycin, itaconic acid, and others. Spectacular success has been achieved with penicillin, and some moderate success with itaconic acid. A logical interpretation for these yield increments is that genetically controlled enzyme systems active in normal cells, and which offer an outlet for some of the intermediary compounds of the cell, are eliminated in the mutants, making proportionally more of the intermediates available to the other intact enzyme mechanisms, one of which, on a random basis, happens to be of interest to the investigator. It would be difficult to account for increased synthetic powers on any other basis.

The best evidence in support of metabolic shunts is that, other factors remaining constant, the enzyme saturation can be demonstrated simply by increasing the concentration of carbohydrate. In dilute sugar media, from 0 up to 0.5 to 2.0 per cent sugar depending on conditions, molds usually will yield no organic acids during the phase of active growth. This is an experiment approximating the nutrition of molds in their natural surroundings: adequate minerals of all

kinds, sufficient utilizable N, and very low C/N ratio due to very low carbohydrate supply. Soluble carbohydrate concentration in the soil probably never comes anywhere near 0.5 per cent. Results from such experiments may be adduced as comparable to the happenings in the soil environment. Next comes a sugar concentration range where very small amounts of metabolic products will accumulate, and this becomes larger in proportion to increased sugar concentration up to a maximum of 8 to 15 per cent for most fungi. This parallel accumulation of acids or other products usually is interpreted simply as the effect of carbohydrate concentration. Actually it is more a measure of sugar split products in excess of those required to saturate the enzyme systems involved in the synthesis of protoplasm and in the oxidation to CO_2. Essentially it is "overflow" metabolism.

It is evident the metabolism of the mold in a culture may be quantitatively as well as qualitatively different as the sugar concentration falls as a result of consumption. The final balance of products represents merely the resultant of all the changing processes. The validity of Kluyver and Perquin's (10) observation that clearcut biochemical evaluation of a mold can be made only in a high sugar concentration and for such a short time that the sugar concentration does not change materially is an all too-little appreciated fundamental of mold metabolism.

If now conditions are imposed which alter the content of or capacity of the bottleneck enzymes, it might be expected that corresponding alterations in the amount of split products diverted through shunt reactions would take place. This is actually the case, and it is possible experimentally to vary the intensity of the shunt reactions within wide limits by controlling key enzyme systems. The effect of sugar concentration has already been discussed, and other evidence supports the idea.

An easy demonstration of these points involves the so-called resting cell technique, also referred to as the replacement method (Pilzdecke), incidentally, also first used by Pasteur. For example, under certain conditions of cultivation where low sugar concentration is present in the medium, *Rhizopus nigricans* or almost any other mold, will grow rapidly and synthesize an abundance of cell material and form much CO_2. Careful tests on the culture filtrate fail to reveal the presence of even traces of organic acid. If this "pregrown" mycelium is now placed in a solution containing the same concentration of sugar as originally present in the medium but, except for some $CaCO_3$ as a neutralizing agent, containing no other nutrient material, large quantities of an organic acid, in this case fumaric acid, are formed quickly and accumulate in amounts comprising a substantial portion of the sugar consumed. That is, the identical cells which formed no acids from sugar during growth now form acids abundantly. The situation here is a logical development of the theme given above. During the growth stage, with an abundance of all nutrients essential for the building up of cell substance, the sugar split products are combined with nitrogen, sulfur, and minerals and built up into larger structural and functional components of cell material. In the replacement experiment with sugar solution, the sugar split products cannot

be further converted into protoplasmic materials in conjunction with nitrogen, sulfur and minerals because the latter are absent. Unable to be consumed through normal synthetic or growth channels, the split products are diverted and partially oxidized through supplementary enzyme systems, which happen to give rise to organic acids and, as seen later, possibly other materials. Some CO_2 also is always formed, and doubtless some is converted to intracellular carbohydrate *via* oxidative assimilation.

One recalls in this connection that the amount of organic acid formed per gram of carbohydrate consumed during the early stages of growth of molds always is less than that formed in a corresponding period during the later stages of incubation. Only near the end of maximum growth, i.e., when cell synthesis begins to slow down, are maximum conversion yields obtained, due to diversion of carbohydrate dissimilation through channels of acid formation.

A further striking example in support of the metabolic shunt is provided by the elegant experiment of Beadle, Mitchell and Houlihan (1) in which the enzyme normally acting upon a metabolic intermediate is removed entirely by destroying the gene responsible for synthesis of that enzyme, i.e., creation of a mutant differing from the normal parent only by lack of one specific enzyme. A mutant strain of the mold *Neurospora crassa* was obtained which could not synthesize adenine due to lack of the enzyme essential for the conversion of adenine precursor to adenine. Blocked in its normal outlet, the precursor is now disposed of in a manner apparently totally foreign to a normal strain,—it undergoes polymerization to form a purple pigment which accumulates in the mycelium and medium. To all appearances the organism has acquired a character, yet in reality it is merely forced to use an otherwise latent enzyme system.

Based on the foregoing, one of the best ways to test the latent ability of an organism to accumulate dissimilation products, is to provide it with excess sugar or other substrate and deprive it of one or more other nutrients essential to growth. This applies, of course, only to preformed vegetative cell material. Starting from spores, no growth would occur if an essential element were omitted. One might accomplish similar results by providing only limited amounts of a certain supplementary nutrient so that it quickly becomes exhausted during early growth, then forcing sugar metabolism through the accessory metabolic channels.

Another interesting demonstration of this idea centers around the catalytic effect which trace elements exert on the efficiency of utilization of available carbohydrate by molds. Notable in this respect is zinc, and to a lesser extent iron, manganese, and copper. The exact mechanisms by which these elements participate in mold metabolism are not known, but their overall effects have been established many times as catalyzing the conversion of substrate into cell material.[4] If a few ppm of zinc ion are added to one flask of a zinc-deficient 2 per cent carbohydrate complete-mineral medium and another no-zinc flask of the same medium inoculated with any acid-forming mold, and the cultures analyzed after a suitable growth period of 5 to 10 days, some profound differences are apparent. The zinc containing culture will have synthesized an abundance of

[4] For review see Foster, J. W., 1939. Bot. Rev., **5**, 207–239.

mycelium meanwhile producing no organic acid, or, at most, very small amounts. The no-zinc culture gives the reverse picture, much smaller mycelium development and substantial accumulation of organic acid in the culture filtrate. Also, in the zinc culture, an appreciably larger percentage of carbohydrate carbon ends up as CO_2 as compared to the no-zinc control.

The explanation of these striking differences lies in the rôle of zinc, presumably functioning as a coenzyme, in catalyzing some reaction that permits more complete oxidation and conversion of the carbohydrate into cell material. Remembering that this conversion necessitates utilization of sugar split products, it is evident that zinc functions in some way as a mediator of enzymes involved in the transformation of split products to protoplasm and that the presence of the right amount of zinc is the governing factor in these transformations. In the zinc deficient culture the transformation enzymes can operate only inefficiently, hence the split products are diverted to organic acid forming enzymes.

However, the fully efficient zinc enzymes, of sufficient catalytic power to handle completely the split products from a 2 per cent sugar medium, can themselves become saturated by the split products from a higher sugar concentration (5 to 10 per cent), so that overflow metabolism sets in, and considerable organic acid accumulation takes place in the presence of an amount of zinc-enzyme sufficient to repress acid formation from lower sugar concentrations.

Further experimental confirmation of the metabolic shunt origin of products in mold cultures involves supplying artificially a dose of the precursors of a particular end product during the stage of enzyme saturation, i.e., during active utilization of supra-minimal sugar concentrations. Two Italian investigators (4) did this experiment, adding to cultures of *Aspergillus niger* equimolar quantities of malic and glycolic acids as citric acid precursors in accordance with one of the current schemes for the biological synthesis of citric acid, namely condensation of C_4 and C_2 acids. In every case increased citric acid yields were obtained, as high as 9.28 times the amount formed from the sugar alone, when obviously the citric acid-forming enzymes were still unsaturated and were acting on split products or derivatives diverted from other saturated oxidative or synthetic mechanisms.

Several other examples of shunt metabolism could be cited. The well-known practice of employing poisons for specific enzymes is one. This technique is such a common one that details will not be given except to emphasize that when a poison shifts the balance of products formed by a microörganism, it is in principle, effecting a shunt metabolism. Normal enzymes are inhibited and subsidiary enzymes then come into play with greater intensity than otherwise. One may cite also the accumulation of reduced metabolic products as an aerobic organism is deprived of oxygen. In molds the reduced product generally is ethanol, while under good conditions of aeration, it is not formed, or, more often, in smaller quantities. In the presence of oxygen, C_2 split products, if formed at all, are oxidized *in situ* to CO_2 and H_2O *via* flavoprotein and the cytochrome hydrogen transport system. Deprived of this pathway, in absence of oxygen, C_2 split products function not as hydrogen donors for oxygen, but as hydrogen acceptors from triose, and become reduced to ethanol.

It is understood that shunt reactions are in reality paired reactions which depend not only on a saturated and overloaded enzyme system, but also on a second enzyme system, normally latent or subdued, whose activity becomes manifest or accentuated through the availability of overflow intermediates. Not unexpected, then, would it be to find instances where the latter enzyme system is lacking and, as a consequence, the overflow intermediate is not metabolized through a diversionary route. One would look for the hypothetical intermediate to accumulate, inasmuch as there is no other way out. Several examples of this type could be given. A fine instance of this simplest kind of metabolic block occurs in a strain of *Fusarium lini* in which a natural cocarboxylase deficiency results in a retarded rate of pyruvate decarboxylation as compared to the rate of formation of this acid from carbohydrate, the pyruvate accumulating and being easily isolated from the medium. Addition of thiamine to the culture medium restores the cocarboxylase level essential for maximum efficiency of carboxylase activity, eliminating thereby the enzyme bottleneck and pyruvate no longer piles up in the culture fluid (15).

From all the foregoing it is evident that the ability to form dissimilation products is intimately linked with the processes of cell synthesis and carbon dioxide production. Resolved into mechanisms, the final balance depends on the capacity of the oxidative and cell synthesizing enzymes in relation to the load of carbohydrate split products they have to carry.

MECHANISMS AND POSTULATED INTERMEDIATES

One of the most favored and time honored approaches to the problem of intermediary metabolism is to feed a biochemical system a series of chemicals postulated to occur somewhere between the breakdown of the substrate and the formation of the particular endproduct. If the system utilizes the added compound and produces in reasonable yield the identical endproduct formed from the original substrate, the added substance is considered to be a normal precursor of the endproduct in the pathway from the original substrate. Extensive use has been made of this technique in mold metabolism, particularly in relation to mechanisms of formation of organic acids. Almost invariably the technique has been to employ the supposed intermediate as the only source of carbon in an otherwise complete medium, inoculate the organism and test for the particular endproduct in question after suitable incubation times. Generally preferred is the technique of using washed, preformed mycelium furnished with the suspected compounds alone or with accessory nutrients.

It is not the purpose of this article to judge the general validity or acceptability of this kind of evidence in biochemical work. However in mold metabolism the situation is such as to warrant a few theoretical observations specifically applicable to this field. Despite rather general use of this approach in the study of any one product, be it oxalic acid, kojic acid, citric acid or others, the results so far available are diverse and so conflicting that with few exceptions it is impossible to draw decisive conclusions as to the true mechanisms in question. For example it is, on the surface, astonishing that such opposing data have been obtained per-

taining to the single process of oxalic acid formation in fungi, all with the "added intermediate" technique. Thus some authors get abundant oxalate formation from acetate, others insignificant yields. Some find and propose glycolic and glyoxylic acids as midway between acetate and oxalate especially on the strength of some conversion of these two acids to oxalate. Others maintain oxalate results from a hydrolytic split of oxalacetic acid, the latter resulting from acetate condensation through the C_4 dicarboxylic acid system. Careful experiments by a different worker fail to reveal any oxalate when oxalacetate is fed to the organism; instead this worker excludes acetate from any rôle in the process and postulates instead a hydrolytic fission of 2-keto gluconic acid to yield oxalate. Others believe that acetate is split out of initially formed citric acid; and next are the experiments in which oxalate is formulated as originating by dehydrogenation of 2 mols of formic acid. Finally no one has offered any mechanism for the extraordinary high yields of oxalate obtainable from peptone solutions. One must remember, too, there is considerable arbitrariness as to whether a yield of the endproduct is of sufficient magnitude to warrant assertion that the tested substance actually is a precursor. In some cases conversion yields of only a few per cent have sufficed to incriminate certain precursors, and yet other workers believe that the bulk of the precursor should eventuate as the product, else the reaction is a secondary side one.

Controversial results like these typify other branches of mold biochemistry. It is illogical, mainly on the basis of comparative biochemistry, to assume the existence of so many different mechanisms for the formation of a single organic acid resulting from carbohydrate breakdown. There must be a flaw in the experimental approach, and a likely one stems from the concept of shunt metabolism.

Worth reiterating here is the view expressed and implicit in the previous section that an organic acid (for example) is formed in quantity from carbohydrate only after the organism has satisfied its primary assimilatory requirements. The precursors of organic acids are surplus over those requirements. Now when a fungus is furnished a hypothetical precursor as the sole carbon source the likelihood is exceedingly strong that a significant portion, if not the bulk of the precursor, goes into the now unsaturated assimilatory or respiratory channel, or both, in which situation the precursor is no longer surplus. And since precursors generally are compounds which would yield integral assimilation building blocks only inefficiently, a large amount of these compounds would undergo consumption and combustion to fulfill these primary needs of the organism, leaving little chance for direct conversion of precursor to product. In such circumstances an actual precursor might be erroneously eliminated from consideration.

It is entirely conceivable that differences in results obtained hitherto by various workers may be explained by the use of different strains of *A. niger*, or other organisms, which on account of strain specificity, vary quantitatively if not qualitatively in the degree and efficiency to which their assimilatory and respiratory requirements are saturated. Strain specificity doubtless explains the prevailing confusion.

To put the experimental method on a basis consistent with theoretical concepts one must perform such experiments under conditions where the complicating assimilatory and respiratory processes are, so to speak, presaturated and hence minimize the importance of these phenomena in the independent conversion of precursor to product. The most logical and efficient way of doing this is to have the organism actively metabolizing carbohydrate before and during the presence of the added precursor. Consumption of precursor now should theoretically be largely via conversion to endproduct. Obviously, optimum conditions would be those where the assimilatory reactions are saturated, and the system forming the particular endproduct unsaturated, so the latter can accomodate added precursor. Hence the carbohydrate concentration should be sub-optimal for maximum product formation when tested alone; indeed, that concentration just beginning to manifest overflow metabolism by the appearance of small or moderate yields of product might be the best one to employ for the precursor additions.

Adoption of such a technique, or at least the principles involved, might provide the means for obtaining more consistent results and in stabilizing what is presently a decidedly unsettled field. Incidentally, it might be pointed out that on theoretical grounds at least one other possibility exists for eliminating the interference of assimilatory mechanisms in preformed mycelium, namely, through selective inhibitions by poisons. A program testing these possibilities is under way in this laboratory.

MAIN PATHWAYS OF CARBOHYDRATE METABOLISM IN MOLDS

Under this heading it is intended to present a rationale which in a general way will serve to coördinate what appear on the surface to be a host of complex and unrelated types of metabolism in the numerous molds so far studied in some detail. If one invokes the precepts of comparative biochemistry first enunciated and brought to bear on microbiology in 1925 by the eminent Dutch microbiologist in Delft, A. J. Kluyver (8, 9), and since continuously espoused in this country by his disciple and former student and associate, C. B. van Niel, one finds it possible to discover a certain uniformity throughout the field of mold metabolism. The very numerous different principal metabolic activities of molds can be looked upon as manifestations of a few main types of metabolic activity. The great majority of them can be considered merely as extensions of the preceding ones so that gradually a series is built up, with comparatively simple examples on one end compounding successively to extreme complexity on the other. Ramifications branch off the main series, to account for the extreme diversity of metabolic types encountered. Viewed in this way, one perceives, in essence, what possibly might be considered as an evolutionary development from simple to complex metabolism, especially since in many cases the logical intermediate steps in the development of the series are known. Or maybe (more likely!) the simple are derived from the complex through successive loss of function or of enzyme systems. No argument is made that the schemes to be presented are evolutionary. The main value of this idea is that is provides a

foundation on which the principles of mold metabolism can be resolved into orderliness.

It may be emphasized at this point that this discussion deals with the biochemical origin in molds of the main kinds of organic compounds generally known to be formed by pure cultures of the organisms growing on media of relatively high carbohydrate content. Commonly they are referred to as "waste" or "excretion" products, though, as seen later, this need not be the correct interpretation. Not included here is the mechanism of formation of vital components of the cell, i.e., protoplasm, nor the mechanism by which CO_2 originates from sugar except where it has a bearing on other points under consideration. Under scrutiny here is the third of the three main fates of carbohydrate carbon, namely, metabolic products, the other two being cell material and CO_2. Only generalized concepts will be given; the various processes are treated in detail in numerous papers and treatises devoted to the individual processes.

The object of the following schematic presentation is to bring out the logical relations which exist among the main products of mold carbohydrate metabolism, and, where possible, to indicate that many of them have intermediary synthetic steps in common. In several instances a product accumulated by one organism represents a simple further transformation of a product formed characteristically by another mold. In the latter, the substance accumulates, due to inability of the fungus to effect further conversion quickly. The further conversion is effected in the former, hence the first substance does not accumulate, but a second one does. In this way it is possible to visualize a common metabolic channel for most fungi, any one differing from others by its ability to carry out one or more additional simple, single step reactions.

It is to be expected that in the more complex of metabolic systems, evidence for intermediates and transformations common to the simpler metabolic system would exist. Wherever investigated, this has been found to be true.

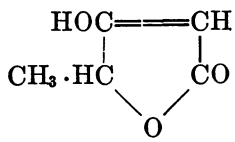
Not only can one find this kind of stepwise metabolic sequence among closely related organisms, but there are numerous instances of the same or similar sequences between distantly related organisms. Seemingly this points to a certain unity of biochemical actions throughout the whole of this group of organisms, a conclusion entirely compatible with Kluyver's generalized concept. There appears to be no general pattern relating taxonomy to biochemical potentialities within the fungi, and this could mean that metabolic offshoots evolved independently of structure. On this basis it is therefore in agreement with expectations that the same major types of biochemical activity are found to occur among widely unrelated groups of fungi.

Thus, most of the Mucorales, and certain penicillia and aspergilli have a preponderant C_2 metabolism, producing from hexose ethanol, acetic acid, with or without oxalic acid.[5] Others carry this C_2 stage through the C_4 dicarboxylic acid stage only, producing mainly fumaric, malic and succinic acids, and this is

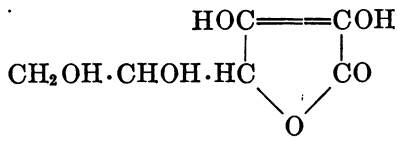
[5] Indeed, available criteria make it likely that in filamentous fungi as a whole the dominant carbohydrate metabolism centers about primary formation of C_2 split products.

typical of the genera *Rhizopus* and *Fusarium* also. C_2 fractions can always be found as intermediates in these processes. In still other mucors, aspergilli and penicillia, as well as other fungi, these C_2 and C_4 compounds are used as precursors of citric acid, which accumulates in large quantities. Yet the C_2 and C_4 intermediates can usually be detected in the medium, accompanying, in small amounts, the major end product, namely citric acid. Though little experimental evidence is available, it is likely that in certain other aspergilli, *Aspergillus itaconicus*, for example, the citric acid or rather its equilibrium product, aconitic acid, functions only as an intermediate, not accumulating but being further converted through a further simple step into itaconic acid by decarboxylation. This is the most logical account of the accumulation of itaconic acid by these organisms.

One is also reminded by this line of reasoning of the simple chemical relations between the 5-membered ring acids produced by *Penicillium charlesii* as revealed by the Raistrick school. These are γ-methyltetronic acid, carolinic acid, carlic acid and carlosic acid. In addition, ethylcarolic acid (terrestric acid) is formed by *Penicillium terrestre*, and the latest stage in the picture as it exists today is the synthesis of ascorbic acid by *Aspergillus niger* (6). All these compounds are differently substituted tetronic acid derivatives, the relation of ascorbic acid to tetronic being as follows:



γ-methyltetronic acid



ascorbic acid

In these cases, it appears that the metabolism is common, the organisms differing in their ability to carry out the final simple transformations. In the case of *P. charlesii* the synthetic sequence is also carried out by a single organism, but the other species mentioned can carry out modifications of this sequence.

Analogous systems exemplifying this principle may be found in the formation of 3,5-dihydroxyphthalic acid and three other derivative acids (C_{10}) by *Penicillium brevi-compactum*, and in the formation of different chemically homologous anthraquinone pigments by different species of *Helminthosporium* (5).

The main theme of the following scheme centers around the formation of split products from carbohydrate, and the type and fate of those split products. Based on this idea the following groupings are possible:

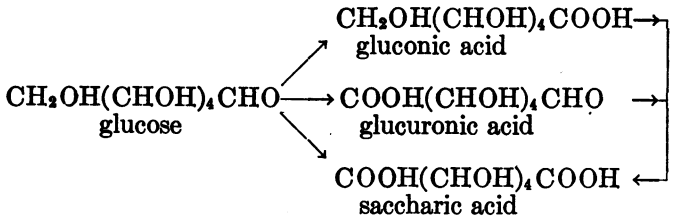
(A) No split products formed during sugar (hexose) utilization. In all these reactions the carbon skeleton of the carbohydrate is left intact.

(1) Gluconic acid and other sugar acids from aldosugars by oxidation of the aldehyde group, including galactonic acid, mannonic acid, xylonic acid, arabonic acid, etc.

(2) Uronic acids, in which the primary alcohol group is converted to a carboxyl: glucuronic acid, etc.

(3) Dicarboxylic sugar acids resulting from oxidation of both the aldehyde and the primary alcohol carbons to carboxyls: saccharic acid, mucic acid, etc.

These processes may be represented as follows:



(B) Split products formed during sugar utilization.

In common with that of all other living systems, the dissimilation of hexose sugars by molds follows uniformly the well-known mechanism of sugar breakdown through the triose or pyruvic acid stage, here referred to as C_3 compounds. And, as in the case of bacterial metabolism where many and diverse metabolic end products are encountered, the nature of the end products depends on how molds dispose of the intermediate C_3 compounds, this in part being a function of the enzyme makeup of any particular organism. In view of its easy transition from C_3 compounds and its extremely important metabolic significance, acetaldehyde (C_2) may also be considered with the C_3 compounds for the moment. Just as in the case of all other living systems, C_3 and C_2 compounds are the key intermediates in the formation of almost all mold metabolic products. The other main influence on the disposition of the C_3 and C_2 compounds is the degree of anaerobiosis *vs.* aerobiosis, or in effect, the availability of oxygen.

Since the origin of C_3 and C_2 compounds lies in dismutation reactions independent of oxygen, the ultimate metabolic products may be considered to have passed through two stages of metabolism, the initial stages, anaerobic or fermentative, and the final, either a continuation of anaerobic reactions or the participation of aerobic reactions, depending on the compound. Often, for the second stage, a mold may possess enzymes capable of effecting both anaerobic and aerobic transformations. In such cases, and similar to most normal cells, the availability of oxygen generally suppresses the so called anaerobic reactions, though not always, *viz.*, lactic acid formation by certain of the Mucorales.

In addition to the above aspects of the fate of the C_3 and C_2 intermediates are two other main features:

These fragments are transformed in various ways without changes in the carbon chain and are left finally still as C_3 and C_2 compounds.

The fragments undergo condensation reactions leading to the accumulation of more complex compounds of higher molecular weight.

The condensation may be pure, involving either only C_3 or C_2 compounds, or, as is likely in some cases, may be mixed, in which C_3 and C_2 compounds may

combine with the other or with condensation products of the other. The condensations may be simple, involving only two or three molecules, or it may be highly multiple, leading to very complex high molecular weight compounds.

I. C_3 split products

(a) Simple conversion: lactic acid, glycerol, pyruvic acid

(b) Condensation:

Two molecules→ Kojic acid[6] (3), hexose sugars, single ring compounds.

Many molecules→Complex ring compounds, pigments, including anthraquinones. Many compounds isolated by Raistrick school.

Of the condensation reactions only kojic acid and hexose sugars have experimental evidence in their support. On the basis of the ring synthesis in kojic acid, the idea is, by analogy, extended to include polycyclic compounds, although no evidence whatsoever is available on the synthesis of these compounds. It should be emphasized that kojic acid is a C_5 ring, whereas many benzene ring type compounds are known to accumulate in mold cultures.

II. C_2 split products

(a) Simple conversion: acetaldehyde, ethanol, acetic acid, ethylacetate, oxalic acid.

(b) Condensation:

Two molecules → (C_4) succinic acid, malic acid, fumaric acid, oxalacetic acid.

$C_4 + C_3 \rightarrow C_7$

or

$C_7 \xrightarrow{-CO_2}$ aconitic acid ⇅ citric acid $\xrightarrow{-CO_2}$ itaconic acid

$C_4 + C_2 \longrightarrow$ aconitic acid

8-9 molecules C_2 → higher fatty acids: stearic, oleic, palmitic, etc.

Several molecules C_2 + Several molecules C_3 → Complex high molecular weight pigments, and other synthetic compounds.

Worthy of mention in connection with the condensation reaction in this section is that the products of primary condensation, which are excreted and accumulate in cultures of some organisms, may, in other organisms, participate in further condensation reactions leading to larger molecular weight compounds. In the

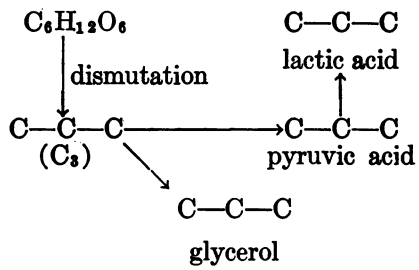
[6] Kojic acid is also claimed to be formed through dehydration and ring closure, producing the γ-pyrone direct from the hexose molecule without primary split products being formed (2, 11).

above listing, citric acid originates from the condensation of a primary condensation product, C_4, and a C_3 or C_2 compound. Similarly, many of the complex pigments and anthraquinones could be interpreted as arising from secondary condensations of the primary condensation rings, namely, simple unicyclic rings. Oxidations, alkyl chain synthesis, etc., all are involved in the building of the final molecule. A scheme based on such ideas has been presented by Tatum (14) to account for the origin of the numerous and diverse complex substances isolated from molds and described by the Raistrick school. Again, this scheme is based on chemical logic, there being, unfortunately, absolutely no experimental evidence available by which to be guided. Nevertheless, this kind of inductive correlation is of great value in coördinating what otherwise might appear to be a confusing mixture of different chemical substances. The value of Tatum's sequence is that it affords a credible explanation as to how the simpler of Raistrick's substances, isolated from certain fungi, may be further converted by additional condensations, substituent incorporations, oxidations, etc., into the more complex structures isolated from other fungi. An analogy to the origin of citric-aconitic acids is apparent.

It will be noted that there are two mechanisms for synthesis of C_4 compounds, namely, $C_2 + C_2$ and $C_3 + C_1$. Probably another mechanism, yet to be demonstrated in molds, is their origin from α-ketoglutaric acid (C_5) by decarboxylation: $C_5 \xrightarrow{-CO_2} C_4$.

Carbon Skeleton Transformations Involving C_3 *Compounds*

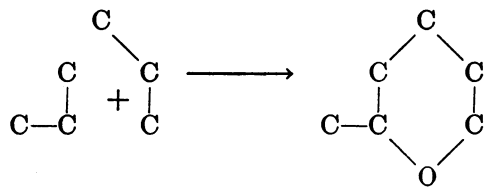
(a)



(b) C—C—C + CO_2 ⟶ C—C—C—C oxalacetic acid

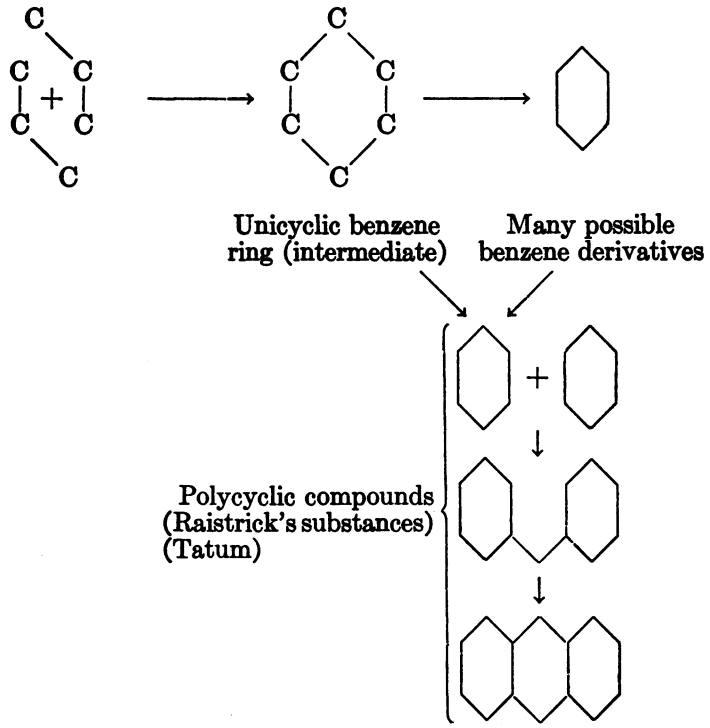
(c) C—C—C + C—C—C → C—C—C—C—C—C → (C—C—C—C—C—C)$_n$
hexose polysaccharide

(d)



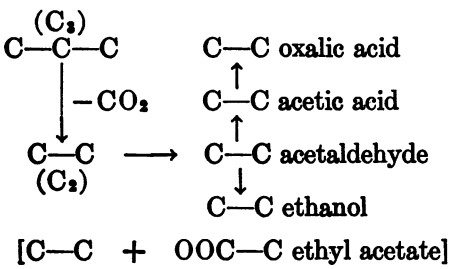
kojic acid

(e)



Carbon Skeleton Transformations Involving C_2 *Compounds*

(f)

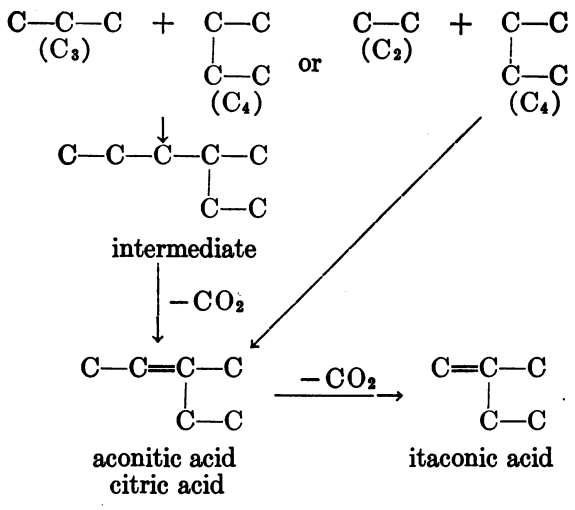


(g) C—C + C—C → C—C—C—C succinic acid
C—C—C—C fumaric acid
C—C—C—C malic acid
C—C—C—C oxalacetic acid

(h) $(C—C)_8 \rightarrow C_{16}$ straight chain; palmitic acid

(i) $(C—C)_9 \rightarrow C_{18}$ stearic, oleic, linoleic acids

(j)



RESERVE STORAGE MATERIALS?

It is obvious that throughout the infinite variety of molds studied, on account of their diversified enzyme makeups, the products of overflow or shunt metabolism will be equally diverse. Not unexpected is the finding that some are water insoluble, or are non-diffusible through the cell membranes, and, consequently, are deposited within cells and accumulate there in substantial quantities, amounting in some cases up to 50 per cent of the total weight of the cell material. Considered in this light, metabolically speaking, there are two main types of metabolic shunt products, those soluble and diffusible in water, and those which are not. Fats, polysaccharides, complex pigments, and compounds of the Raistrick type which are deposited in and accumulate in mycelium are insoluble shunt products whereas organic acids, alcohols, pigments and other low molecular weight compounds are soluble shunt products.

There is no more reason for considering fat and polysaccharide depositions as reserve or storage products, than the carbohydrates and organic acids formed and which accumulate outside the cells. One might even include the complex benzenoid pigments found by the Raistrick school to comprise 30 to 40 per cent of the cell material. The principal arguments advanced in support of the "reserve material" hypothesis are two: they are laid down during luxury carbohydrate nutrition of the organism; and they disappear, by cellular oxidation, during starvation conditions, supposedly serving as a source of energy and carbon.

The first argument is equally true of all products, including organic acids, accumulated by molds either within or outside the cell. There is ample evidence also that the second argument is not peculiar to the intracellular accumulations. The observation is a rather general one that extracellular accumulations also tend to be utilized by the organism after exhaustion of the primary energy source, namely carbohydrate. Yields of most, if not all, organic acids tend to fall after reaching a maximum coinciding with depletion of the sugar. Just as fat dis-

appearance is slow and gradual, so is the attack on the initially accumulated soluble organic acids and carbohydrates.

In the final analysis this is simply a reflection of the ability stated above of fungi eventually to oxidize completely the original available substrate to CO_2 and H_2O, besides synthesize cell material. So long as the organism is surfeited with easily consumed carbohydrate, the attack on shunt byproducts is deferred. Relieved of their saturation by the preferentially decomposed sugar, the dismutation enzymes now proceed with the slower degradation of the initially formed products of deranged metabolism.

Actually, the rate at which accumulated fats are attacked by the mold is so slow that it is difficult to see how they could be of value to the organism as a "reserve product." No evidence is available, but it seems possible that intracellular accumulations of the complex compounds, pigments, benzenoid compounds, etc., also might be further attacked and slowly consumed, provided an abundance of oxygen is available. Functionally speaking, these compounds could very well be considered in a class with other mold products. Thus, extracellular accumulations of metabolic products would have to be considered as storage or reserve products by the same interpretation that intracellular materials are. Such a conclusion seems untenable.

More acceptable are the ideas presented above which lead to the conclusion that all such compounds happen to be subject to degradation by the mold irrespective of their location. A water soluble, diffusible compound is by ordinary concepts just as available to the cell as insoluble fat in a vacuole. The attack (and consumption) of these accumulated products probably never begins until the organism exhausts its more easily attacked and preferred energy source:—carbohydrate.

This article could not be complete without my pointing out that I commenced writing it almost to the day ten years after I came under the tutelage of Professor S. A. Waksman at New Brunswick, New Jersey. He is responsible for this article, for he first introduced me to this general subject, acquainted me with its lure and potentialities, urged me to make it my following, and has since, by virtue of his sagacity, zeal and intimate personal friendship and counsel continuously through these years, unwittingly shaped my scientific outlook and extended my scope. For all this, and with appreciation, I dedicate to him this article on one of his favorite subjects.

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